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# The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells

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Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation (BMT), and damage to the gastrointestinal (GI) tract plays a critical role in amplifying systemic disease. Intestinal stem cells (ISCs) play a pivotal role not only in physiological tissue renewal but also in regeneration of the intestinal epithelium after injury. In this study, we have discovered that pretransplant conditioning regimen damaged ISCs; however, the ISCs rapidly recovered and restored the normal architecture of the intestine. ISCs are targets of GVHD, and this process of ISC recovery was markedly inhibited with the development of GVHD. Injection of Wnt agonist R-spondin1 (R-Spo1) protected against ISC damage, enhanced restoration of injured intestinal epithelium, and inhibited subsequent inflammatory cytokine cascades. R-Spo1 ameliorated systemic GVHD after allogeneic BMT by a mechanism dependent on repair of conditioning-induced GI tract injury. Our results demonstrate for the first time that ISC damage plays a central role in amplifying systemic GVHD; therefore, we propose ISC protection by R-Spo1 as a novel strategy to improve the outcome of allogeneic BMT.

An important aspect of cancer therapy is maintaining a fine balance between the use of chemoradiotherapy doses high enough to kill tumor cells and doses low enough to prevent damage to normal tissue. The gastrointestinal (GI) epithelium and BM are the most rapidly self-renewing tissues in adults and are therefore susceptible to cytotoxic exposure, showing a rapid expression of damage. Damage to these tissues is a dose-limiting and potentially lethal toxicity of chemoradiotherapy used to treat cancer patients. Allogeneic hematopoietic stem cell transplantation (SCT) is a curative therapy for hematologic malignancies that works by delivering healthy hematopoietic stem cells to replace BM destroyed by the high-dose chemoradiotherapy (pretransplant conditioning); however, this process is complicated by regimen-related toxicity against other tissues, particularly in the GI tract.

Graft-versus-host disease (GVHD), a major and devastating complication of allogeneic SCT,

is a complex process involving donor T cell responses to host antigens and the dysregulation of inflammatory cytokine cascades (Hill et al., 1997; Hill and Ferrara, 2000; Teshima et al., 2002a; Ferrara et al., 2003). Increasing evidence from experimental and clinical SCT suggests that conditioning-mediated GI tract damage plays a central role in amplifying GVHD by propagating its cytokine storm characteristics (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). Intestinal epithelial cells are continuously regenerated from intestinal stem cells (ISCs), which are key to the regeneration of damaged intestinal epithelium (Battle et al., 2002; Pinto et al., 2003; Barker et al., 2007, 2008). However, the dynamic process of damage and repopulation of ISCs, which play a pivotal

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Abbreviations used: BMT, BM transplantation; GI, gastrointestinal; GVHD, graft-versus-host disease; ISC, intestinal stem cell; R-Spo1, R-spondin1; SCT, stem cell transplantation; TBI, total body irradiation; TCD, T cell depleted.

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role in the competitive race between tissue damage and restoration during conditioning regimens and GVHD, is not well understood.

Wnt signaling plays a critical role in the regulation of intestinal epithelial cell proliferation during their maturation or regeneration (Batlle et al., 2002; Pinto et al., 2003; Reya and Clevers, 2005; Barker et al., 2008). R-spondin1 (R-Spo1) is a potent activator of the Wnt signaling pathway. It relieves the Dickkopf-1 inhibition imposed on the Wnt signaling pathway and thereby increases levels of the Wnt pathway coreceptor low-density lipoprotein receptor–related protein-6 on cell surface (Kim et al., 2005; Binnerts et al., 2007). We have previously shown that human R-Spo1 transgenic mice had a marked thickening of the mucosa and displayed crypt epithelial hyperplasia (Kim et al., 2005). Injection of human R-Spo1 induced rapid onset of crypt cell proliferation in the intestine of normal mice through  $\beta$ -catenin stabilization and subsequent transcriptional activation of target genes (Kim et al., 2005). Thus, injection of R-Spo1 protected mice from chemotherapy- or radiation-induced colitis by stimulating mucosal regeneration and restoring intestinal architecture (Kim et al., 2005; Zhao et al., 2007, 2009; Bhanja et al., 2009). However, because of the lack of specific markers for ISCs, it is unclear whether this result was mediated by the direct effect of R-Spo1 on ISCs.

In this study, we investigated the dynamic process of ISC damage and repopulation during the pretransplant conditioning regimen, total body irradiation (TBI), and GVHD. The effects of R-Spo1 on this process were also examined using recently identified markers for ISCs such as *Lgr5* (leucine-rich repeat-containing G protein-coupled receptor 5) and *Olfm4* (Olfactomedin-4; Barker et al., 2007, 2008; van der Flier et al., 2009a,b). *Lgr5* and *Olfm4* mark rapidly cycling crypt base columnar cells, which can give rise to all intestinal epithelial lineages (Barker et al., 2007, 2008; van der Flier et al., 2009a,b). We then tested the hypothesis that protection of ISCs improves the outcome of allogeneic SCT by regulating systemic GVHD using a well-characterized murine model of MHC-mismatched, haploidentical BM transplantation (BMT).

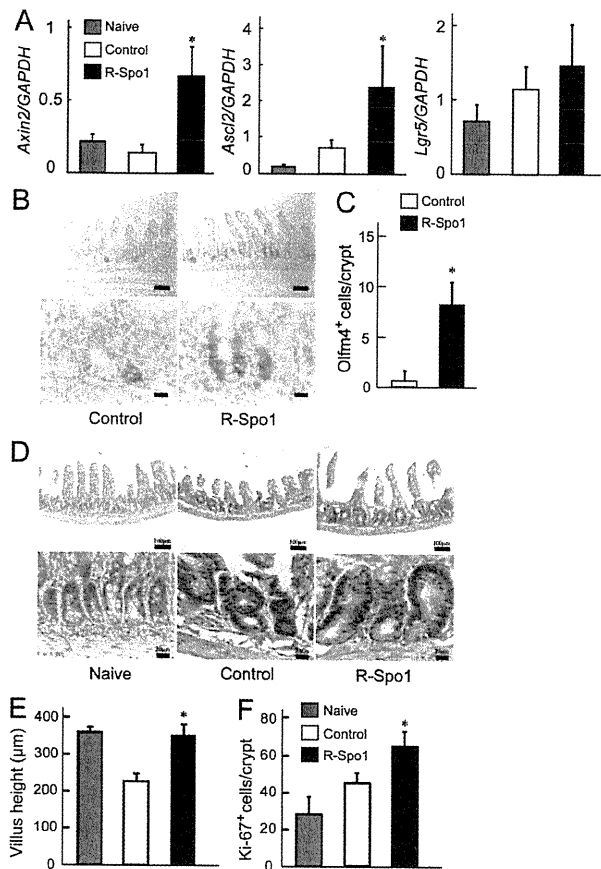
## RESULTS

### R-Spo1 protected against radiation-induced colitis by stimulating proliferation of ISCs through the Wnt signaling pathway

We first studied the effect of R-Spo1 on the expression of Wnt target genes in the small intestine using quantitative real-time PCR. Injection of R-Spo1 (200  $\mu$ g/day) over 3 d significantly up-regulated the expression of Wnt target genes, including *Axin2*, *Ascl2* (*Achaete scute-like 2*), and *Lgr5* (Fig. S1 A). We noted an elongation of villi with an increased number of *Olfm4*<sup>+</sup> ISCs in the crypts of R-Spo1–treated animals (Fig. S1, B and C). Ki-67 immunostaining also showed crypt hyperplasia paralleling an increased number of Ki-67<sup>+</sup> cycling cells in the crypts (Fig. S1, D and E).

Next, we evaluated the effect of R-Spo1 administration on the process of mucosal regeneration after TBI. According to

our preliminary experiments (unpublished data), mice irradiated with 15 Gy TBI on day 0 were intravenously injected with 200  $\mu$ g R-Spo1 once daily from day –3 to –1 and from day 1–3. The real-time PCR analysis of the small intestine harvested 6 h after the final administration of R-Spo1 showed up-regulated expression of *Axin2* and *Ascl2* in R-Spo1–treated mice (Fig. 1 A). The *Olfm4*<sup>+</sup> cell population was significantly greater in R-Spo1–treated mice than in controls on day 3 (Fig. 1, B and C); as a result, radiation colitis characterized

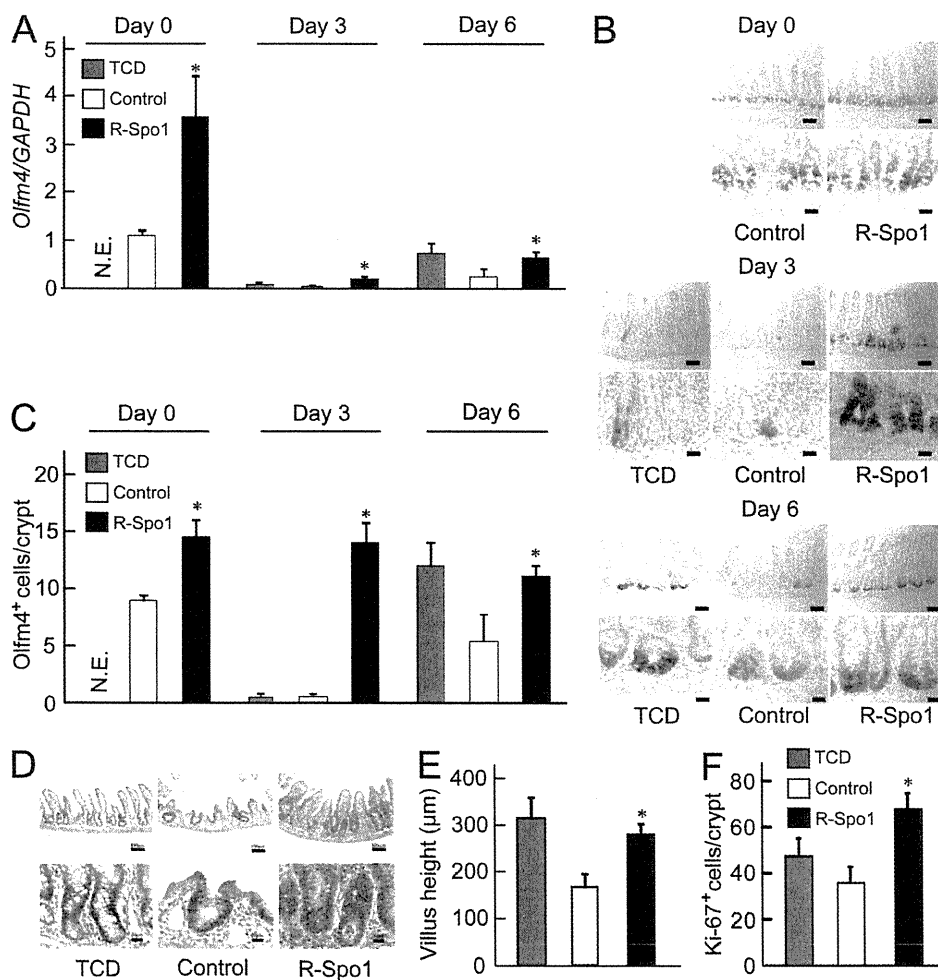


**Figure 1. R-Spo1 protected against radiation-induced colitis by enhancing proliferation of ISCs via the Wnt signaling pathway.** B6D2F1 mice irradiated with 15 Gy TBI on day 0 and intravenously injected with R-Spo1 (200  $\mu$ g/day) or control from day –3 to –1 and day 1–3. The small intestine was harvested 6 h after the final administration of R-Spo1 for quantitative real-time PCR analysis and in situ hybridization, and 24 h later for immunohistochemistry. (A) Quantitative real-time PCR analysis of *Axin2*, *Ascl2*, and *Lgr5* transcripts normalized to those of GAPDH (naive,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$ ). (B) In situ hybridization for *Olfm4* on representative crypts. (C) Quantification of *Olfm4*<sup>+</sup> cells per crypt ( $n = 4$  per group). (D) Ki-67 staining of the terminal ileum. (E) Villus height of the terminal ileum (naive,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$ ). (F) Quantification of Ki-67<sup>+</sup> cells per crypt (naive,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$ ). Data are representative of two independent experiments and are shown as means  $\pm$  SD. \*,  $P < 0.05$  compared with control. Bars: (B and D, top row) 100  $\mu$ m; (B and D, bottom row) 20  $\mu$ m.

by blunting of villi was significantly reduced in R-Spo1-treated animals (Fig. 1, D and E). We also noted crypt hyperplasia and an increased number of Ki-67<sup>+</sup> cycling cells in the crypt on day 4 (Fig. 1, D and F). These results extend our previous observations regarding R-Spo1-mediated mitogenic effects on the intestinal epithelium (Kim et al., 2005) by documenting the effects of R-Spo1 on ISCs.

**R-Spo1 protected against ISC damage after allogeneic BMT**  
GI tract damage is much more severe in allogeneic SCT than in autologous or syngeneic SCT because of the additional detrimental effects of GVHD on the GI tract. However, it remains to be elucidated whether GVHD targets ISCs that are

crucial for the regeneration of damaged intestinal epithelium and also how the damage and repopulation of ISCs affects the process of mucosal injury and regeneration after allogeneic BMT. To address these issues, lethally irradiated B6D2F1 mice were transplanted with  $5 \times 10^6$  T cell-depleted (TCD) BM cells with or without  $2 \times 10^6$  T cells from MHC-mismatched C57BL/6 (B6) or B6-Ly5.1 donors on day 0. Small intestines were harvested from mice on day 0 before TBI and on days 3 and 6 after BMT, and quantitative real-time PCR and in situ hybridization were performed to determine the kinetics of loss and repopulation of *Olfm4*<sup>+</sup> ISCs. R-Spo1 was injected from day -3 to -1 and day 1-3 after BMT. TCD animals and control-treated allogeneic animals served as non-GVHD



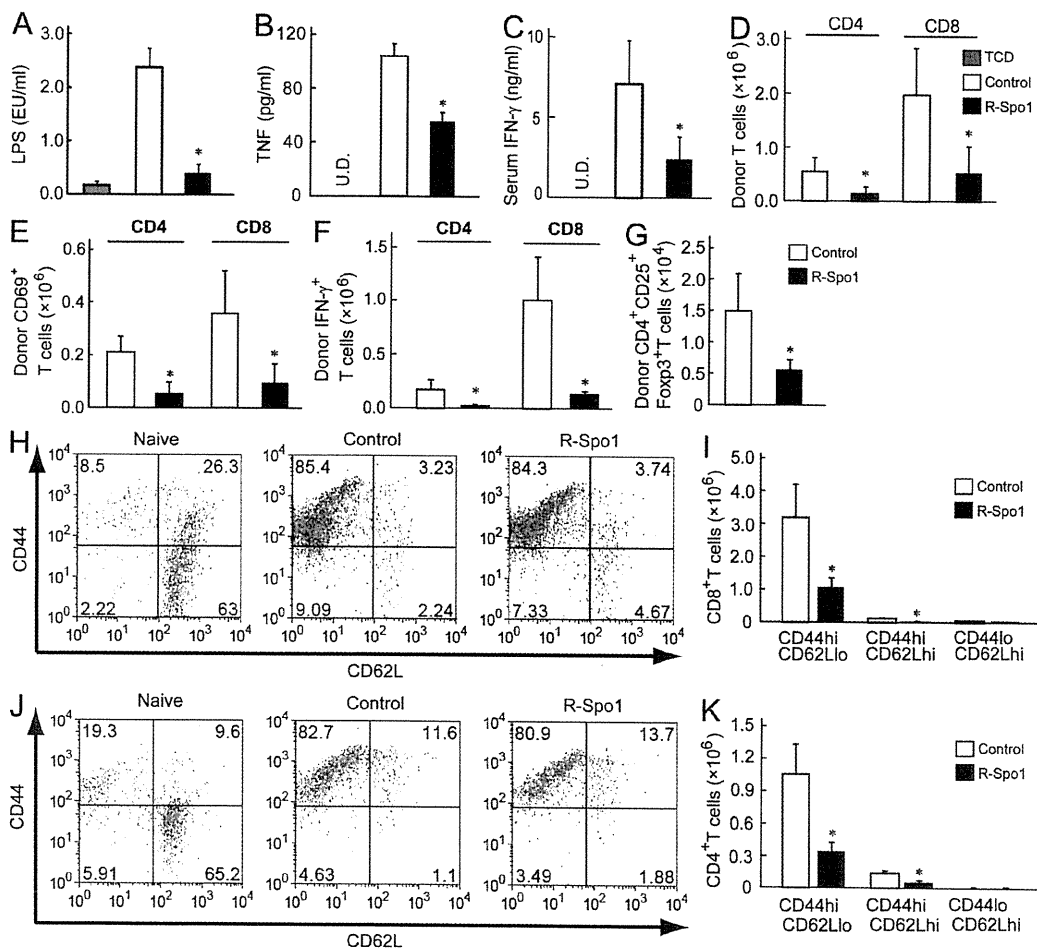
**Figure 2. R-Spo1 enhanced repopulation of ISCs after allogeneic BMT.** Lethally irradiated B6D2F1 mice were transplanted with  $5 \times 10^6$  TCD BM with or without  $2 \times 10^6$  T cells from B6 donors on day 0. R-Spo1 (200 µg/day) or control was intravenously injected from day -3 to -1 and day 1-3 after BMT. Small intestines were harvested on day 0 (before TBI) and days 3 and 6. (A) Quantitative real-time PCR analysis of *Olfm4* transcripts normalized to those of GAPDH (TCD,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$  per time point). (B) In situ hybridization of *Olfm4* on representative crypts. (C) Quantification of *Olfm4*<sup>+</sup> cells per crypt (TCD,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$  per time point). (D) Ki-67 staining of the terminal ileum harvested on day 6. (E) Villus height of the terminal ileum was measured as described in Fig. 1 E using the slides in D (TCD,  $n = 3$ ; control,  $n = 5$ ; R-Spo1,  $n = 5$ ). (F) Quantification of Ki-67<sup>+</sup> cells per crypt (TCD,  $n = 3$ ; control,  $n = 5$ ; R-Spo1,  $n = 5$ ). Data are representative of two independent experiments and are shown as means  $\pm$  SD. \*,  $P < 0.05$  compared with control. Bars: (B and D, top rows) 100 µm; (B and D, bottom rows) 20 µm.

controls and GVHD controls, respectively. In both groups, *Olfm4* expression levels significantly decreased in the small intestine on day 3 (Fig. 2 A). In TCD animals, *Olfm4* expression recovered to normal levels on day 6, whereas in allogeneic controls, it remained low. In contrast, *Olfm4* expression levels in R-Spo1-treated animals were significantly higher on and after day 0 in comparison with allogeneic controls (Fig. 2 A). These results were further confirmed by in situ hybridization analysis of *Olfm4* transcripts in the small intestine. The *Olfm4*<sup>+</sup> ISC population significantly decreased in both TCD and allogeneic controls on day 3 (Fig. 2, B and C). On day 6, *Olfm4*<sup>+</sup> cells were fully repopulated in TCD animals, and their numbers were significantly higher than their numbers in allogeneic controls. These results demonstrate that TBI injures ISCs

and that the process of ISC repopulation is inhibited in GVHD. In R-Spo1-treated animals, the number of *Olfm4*<sup>+</sup> cells was consistently higher before and after BMT than that in allogeneic controls. Villous atrophy was severe in allogeneic controls on day 6, whereas injection of R-Spo1 resulted in crypt hyperplasia with an increased number of Ki-67<sup>+</sup> cycling cells in the crypts and dramatically ameliorated GI tract damage (Fig. 2, D–F). R-Spo1 treatment before TBI thus expanded the ISC pool and minimized intestinal damage.

**R-Spo1 suppressed inflammatory cytokine cascades and donor T cell activation after allogeneic BMT**

We then tested the hypothesis that protection of ISCs against TBI regulates systemic GVHD and improves the outcome of



**Figure 3. R-Spo1 regulated activation of inflammatory and cellular effectors in GVHD.** Lethally irradiated B6D2F1 mice were transplanted with  $5 \times 10^6$  TCD BM cells with or without  $2 \times 10^6$  T cells from B6-Ly5.1 (CD45.1<sup>+</sup>) donors on day 0. R-Spo1 (200  $\mu$ g/day) or control was intravenously injected from day -3 to -1 and day 1-3 after BMT. Serum samples and splenocytes were obtained 5-7 d after BMT (TCD,  $n = 3$ ; control,  $n = 5$ ; R-Spo1,  $n = 5$ ). (A–C) Serum levels of LPS (A), TNF (B), and IFN- $\gamma$  (C) are shown. (D–G) Numbers of donor (CD45.1<sup>+</sup>) T cells (D), CD69<sup>+</sup> donor T cells (E), IFN- $\gamma$ <sup>+</sup> donor T cells (F), and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T reg cells (G) in spleen are shown. (H–K) Flow cytometric analysis and enumeration of T cell subsets in spleen. Numbers represent the percentage of cells in the dot plot quadrants. CD8<sup>+</sup> T cells (H and I) and CD4<sup>+</sup> T cells (J and K) are shown. Data are representative of two independent experiments and are shown as means  $\pm$  SD. \*,  $P < 0.05$  compared with control. U.D., undetectable.

allogeneic SCT. Serum LPS levels are increased during GVHD and correlate with GI tract damage (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). LPS has been shown to stimulate production of excessive inflammatory cytokines such as TNF that are implicated in the pathogenesis of GVHD (Nestel et al., 1992; Hill et al., 1997; Hill and Ferrara, 2000; Cooke et al., 2001; Teshima et al., 2002a; Ferrara et al., 2003). LPS and TNF levels were markedly increased in allogeneic controls but were significantly reduced in R-Spo1-treated animals (Fig. 3, A and B), suggesting that the fortification of GI mucosal barrier functions by R-Spo1 suppresses subsequent inflammatory cascades in GVHD. We also investigated the effect of R-Spo1 on allogeneic donor T cell responses. Serum levels of IFN- $\gamma$ , a hallmark of systemic T cell responses in GVHD, were significantly lower in R-Spo1-treated mice than in controls (Fig. 3 C). Donor T cell expansion (Fig. 3 D) and activation, as determined by CD69 expression (Fig. 3 E), and intracellular IFN- $\gamma$  (Fig. 3 F) were also significantly reduced in R-Spo1-treated mice. Recent studies have shown that Wnt signaling can modulate adoptive immunity by enhancing regulatory T cell (T reg cell) survival and inducing CD4<sup>+</sup> T cell anergy, as well as by regulating effector CD8<sup>+</sup> T cell development and promoting memory CD8<sup>+</sup> T cell generation (Ding et al., 2008; Gattinoni et al., 2009). However, in our study, the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T reg cells in the spleen was significantly less in R-Spo1-treated mice than in controls (Fig. 3 G), and the ratios of effector to memory CD8<sup>+</sup> (Fig. 3, H and I) and CD4<sup>+</sup> T cells (Fig. 3, J and K) were similar between R-Spo1-treated mice and controls.

To further confirm whether the reduction in donor T cell activation after BMT was caused by the direct effect of R-Spo1 on T cells, we investigated the effect of R-Spo1 on T cells *in vivo* and *in vitro*. Administration of R-Spo1 over 3 d had no effect on the number of T cells in naive mice (Table I),

**Table I.** Brief administration of R-Spo1 had no effects on immunophenotype

Immunophenotype	+Control	+R-Spo1
<b>Spleen</b>		
CD4 <sup>+</sup>	20.7 ± 4.2	17.4 ± 2.0
CD8 <sup>+</sup>	14.3 ± 2.5	11.9 ± 0.9
CD4 <sup>+</sup> Foxp3 <sup>+</sup>	2.46 ± 0.63	2.46 ± 0.34
B220 <sup>+</sup>	58.5 ± 10.9	57.8 ± 2.9
<b>Mesenteric lymph node</b>		
CD4 <sup>+</sup>	4.3 ± 0.7	4.9 ± 0.2
CD8 <sup>+</sup>	3.5 ± 0.7	4.2 ± 0.4
CD4 <sup>+</sup> Foxp3 <sup>+</sup>	0.65 ± 0.14	0.70 ± 0.09
<b>Thymus</b>		
CD4 <sup>+</sup> CD8 <sup>+</sup>	96.2 ± 16.9	91.6 ± 19.8
CD4 <sup>+</sup>	8.4 ± 1.0	8.6 ± 2.1
CD8 <sup>+</sup>	3.4 ± 0.3	3.6 ± 0.6

B6 mice were intravenously injected with R-Spo1 (200  $\mu$ g/day) or control for 3 d, and the spleen, mesenteric lymph nodes, and thymus were harvested 6 h later. Cell numbers are shown as mean  $\pm$  SD ( $\times 10^6$ ). Data are representative of two independent experiments ( $n = 4$  for +control and +R-Spo1).

and the addition of R-Spo1 to culture did not affect *in vitro* T cell responses to alloantigens or anti-CD3 cross-linking (Fig. S2, A and B). Furthermore, R-Spo1 addition to culture affected neither the proliferation nor the generation of effector and memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells in response to anti-CD3 cross-linking *in vitro* (Fig. S2, C–F).

#### Brief administration of R-Spo1 ameliorated systemic GVHD

We studied two lethal doses of TBI, 12 and 15 Gy, for their effects on GVHD. At both TBI doses, TCD controls showed 100% survival. All allogeneic controls receiving 15 Gy TBI died by day 40, whereas those receiving 12 Gy TBI displayed 7% survival at day 90 (Fig. 4 A). The TBI dose thus significantly correlated with GVHD mortality, as has been shown previously (Hill et al., 1997). In R-Spo1-treated animals, GVHD mortality was significantly reduced in experiments with 12 Gy TBI and delayed in those with 15 Gy TBI (Fig. 4 A) and reduced GVHD severity as assessed by clinical GVHD scores (Teshima et al., 2002a) in surviving animals (Fig. 4 B). Target organs, including the small intestine, liver, skin, and thymus, were then evaluated for signs of GVHD after allogeneic BMT after 12 Gy TBI. The small intestine and liver samples were harvested 1 wk after BMT, whereas skin and thymus samples were obtained 7 wk after BMT. GVHD-mediated thymic atrophy, characterized by a reduction in the numbers of CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes, was significantly restored in R-Spo1-treated mice (Fig. 4 C). Pathological analysis of the small intestine, liver, and skin showed almost normal architecture in TCD controls (Fig. 4 D). In contrast, allogeneic controls showed severe blunting of villi and inflammatory infiltration, whereas R-Spo1-treated mice showed significant restoration of the small intestinal villous architecture with little inflammatory infiltration. Liver histology of allogeneic controls revealed mononuclear cell infiltration in bile ducts and portal triads (Fig. 4 D, arrowheads), whereas these changes were less prominent in R-Spo1-treated mice. Lesser lymphocyte infiltration was observed in the skin of R-Spo1-treated mice compared with that of allogeneic controls. GVHD pathology scores in each organ were significantly lower in R-Spo1-treated mice than those in controls (Fig. 4, E–G). Flow cytometric analysis of the spleens on day 35 displayed complete donor chimerism (99.9  $\pm$  0.1%), ruling out mixed chimerism as a cause of the reduced GVHD. These results demonstrate that brief administration of R-Spo1 modulates not only intestinal but also systemic GVHD.

Next, we studied how the scheduling of R-Spo1 administration could influence the outcome of allogeneic BMT after 15 Gy TBI. Administration of R-Spo1 from day –3 to –1 and day 1–3 significantly prolonged survival. These beneficial effects were not observed when R-Spo1 was injected only once after BMT from day 1–6 after 15 Gy TBI (Fig. 4 H). When R-Spo1 was administered only once before TBI from day –6 to –1, early GVHD mortality was reduced; however, survival was not prolonged. These results suggest that R-Spo1 injection before TBI is mandatory and that posttransplant administration of R-Spo1 results in maximum reduction of GVHD.

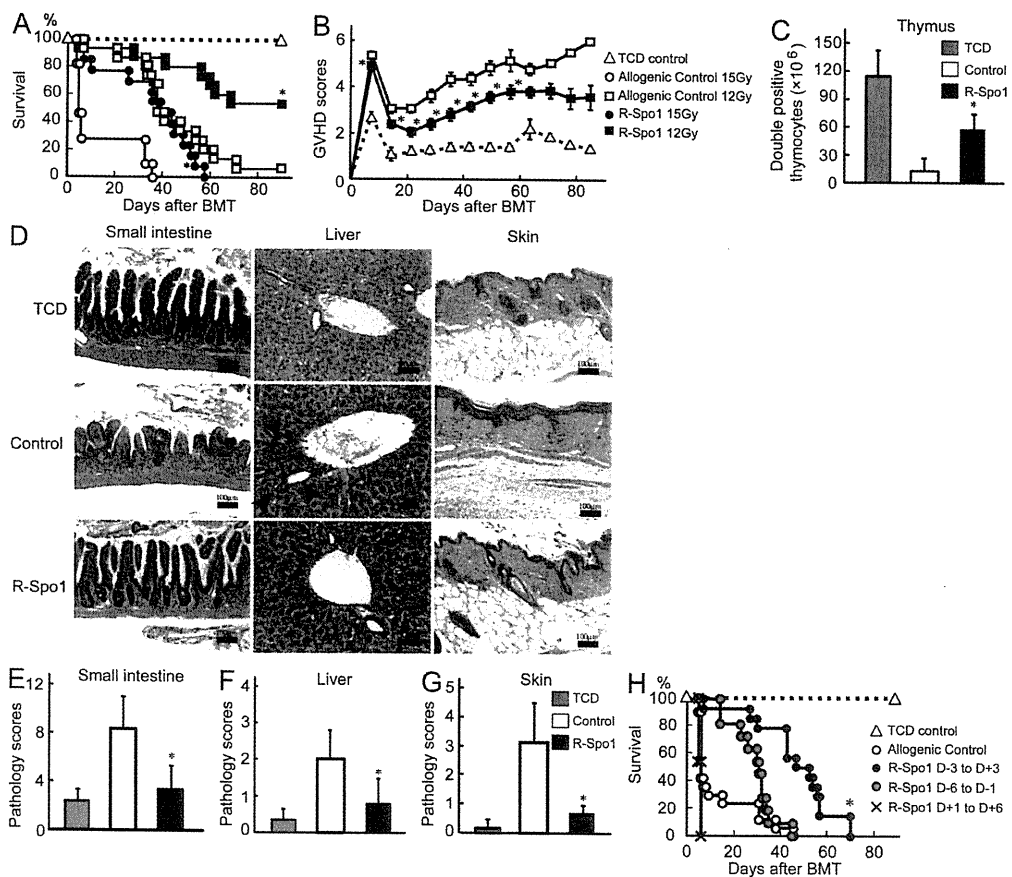
**R-Spo1 regulates GVHD by a mechanism dependent on repair of radiation-induced gut injury**

To confirm that R-Spo1 ameliorated systemic GVHD by a mechanism dependent on repair of radiation-induced GI tract damage, the effects of R-Spo1 were evaluated in the same BMT model without conditioning, as previously described (Mori et al., 1998). Unirradiated B6D2F1 mice were intravenously injected with  $12 \times 10^7$  splenocytes from MHC-mismatched B6 or B6-Ly5.1 donors on day 0. In this model, cytopenia mediated by donor T cell attack of BM is the primary cause of death in GVHD (Via et al., 1987). Injection of R-Spo1 did not impact the mortality or morbidity caused by GVHD (Fig. 5, A and B), donor T cell expansion (Fig. 5 C),

thymic GVHD (Fig. 5 D), GVHD-associated cytopenia (Fig. 5 E), or donor cell engraftment ( $99.7 \pm 0.4\%$  in controls and  $99.9 \pm 0.0\%$  in R-Spo1-treated mice on day 60).

**DISCUSSION**

Intestinal GVHD is characterized by severe villous atrophy and crypt degeneration. It has been suggested that crypt cell degeneration is one of the initial lesions of intestinal GVHD (Sale et al., 1979; Epstein et al., 1980; Mowat and Socie, 2004). ISCs reside in the intestinal crypts and play a pivotal role in both physiological tissue renewal and regeneration of the intestinal epithelium after injury. However, the identity of cells within the crypts (primary targets in GVHD) has been an



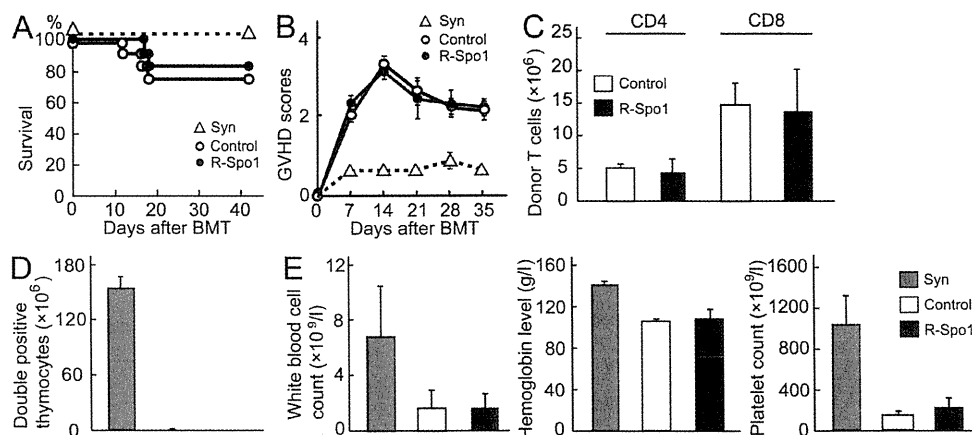
**Figure 4. R-Spo1 modulated systemic GVHD.** B6D2F1 mice were transplanted with  $5 \times 10^6$  TCD BM cells with or without  $2 \times 10^6$  T cells from B6 donors on day 0 after 15 or 12 Gy TBI. R-Spo1 (200  $\mu$ g/day) or control was injected from day -3 to -1 and day 1-3 after BMT. (A and B) Survival (A) and clinical GVHD scores (B; means  $\pm$  SE) are shown. TCD non-GVHD controls ( $n = 6$ ), allogenic controls with 15 Gy ( $n = 11$ ) or 12 Gy TBI ( $n = 15$ ), and R-Spo1 with 15 Gy ( $n = 13$ ) or 12 Gy TBI ( $n = 15$ ) are shown. Data from three independent experiments were combined. (C) Numbers of CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes 7 wk after BMT (TCD,  $n = 3$ ; control,  $n = 5$ ; R-Spo1,  $n = 5$ ). Data are representative of two similar experiments and are shown as means  $\pm$  SD. (D) Representative histological findings of the small intestine, liver, and skin. Arrowheads indicate mononuclear cell infiltration in bile ducts and portal triads. Bars: (left and right) 100  $\mu$ m; (middle) 50  $\mu$ m. (E-G) Pathology scores of the small intestine (E) and liver (F) harvested on day 7 and those of the skin (G) harvested 7 wk after BMT after 12 Gy TBI (TCD,  $n = 3$ ; control,  $n = 5$ ; R-Spo1,  $n = 5$ ). Data are representative of two similar experiments and are shown as means  $\pm$  SD. (H) R-Spo1 was intravenously injected for six doses at different schedules after 15 Gy TBI and BMT. Survival after BMT: TCD non-GVHD controls ( $n = 5$ ), allogenic controls ( $n = 17$ ), and R-Spo1 day -3 to -1 and day 1-3 (D-3 to D+3;  $n = 11$ ), day -6 to -1 (D-6 to D-1;  $n = 14$ ), or day 1-6 (D+1 to D+6;  $n = 11$ ) are shown. Data from three independent experiments were combined. \*,  $P < 0.05$  compared with allogenic controls.

enigma because of the lack of specific markers. In this study, we discovered that pretransplant TBI damaged *Olfm4*<sup>+</sup> ISCs in the crypts; however, the ISCs rapidly recovered and restored the normal architecture of the small intestine within 1 wk. With development of acute GVHD, the process of ISC recovery was inhibited, and prolonged and profound intestinal damage was induced after allogeneic BMT. These observations are well in line with those from a previous study of sequential rectal biopsies from patients undergoing allogeneic BMT (Epstein et al., 1980). Severe crypt degeneration was noted in all biopsies taken soon after BMT, probably because of the conditioning regimen. These changes persisted when acute GVHD was present but disappeared in patients who did not show clinical evidence of GVHD (Epstein et al., 1980). The current study thus affirms the long-held assumption that ISCs may be targets for immune responses associated with GVHD (Sale et al., 1979; Epstein et al., 1980; Mowat and Socie, 2004).

We have previously demonstrated that R-Spo1 induces rapid onset of epithelial proliferation in the intestine by stimulating Wnt signaling and protects against chemotherapy-induced colitis (Kim et al., 2005). However, owing to the lack of specific ISC markers it was unclear whether this effect was caused by the direct effect of R-Spo1 on ISCs. The current study shows that administration of R-Spo1 up-regulates the expression of Wnt target genes such as murine *Axin2*, *Ascl2*, and *Lgr5*. *Ascl2* is a critical transcriptional factor involved in controlling the fate of ISCs in adults (van der Flier et al., 2009b), and *Lgr5* marks ISCs (Barker et al., 2007, 2008). *Olfm4* is not a Wnt target gene but a highly specific and robust marker for *Lgr5*<sup>+</sup> ISCs (van der Flier et al., 2009a,b). We found that R-Spo1 stimulated proliferation of *Olfm4*<sup>+</sup> ISCs, thus taking further the observations from our previous study

(Kim et al., 2005) and confirming recent observations that R-Spo1 enhances the proliferation of cycling ISCs via the Wnt signaling pathway (Bhanja et al., 2009; Sato et al., 2009).

Administration of R-Spo1 has been shown to mediate protection against radiation colitis, which is evident from studies in mouse models with chemotherapy- or radiation-induced mucositis and gut injury (Kim et al., 2005; Zhao et al., 2007, 2009; Bhanja et al., 2009). Our results suggest that R-Spo1-mediated protection of ISCs could be primarily responsible for the protection of the GI tract from radiation, as has been suggested in a recent study (Bhanja et al., 2009). Furthermore, we have shown that brief administration of R-Spo1 suppresses systemic GVHD after allogeneic BMT by a mechanism dependent on the repair of conditioning-induced GI tract injury. Experimental and clinical studies have suggested that GI tract damage resulting from both pretransplant conditioning regimens and GVHD plays a central role in increasing GVHD severity (Hill et al., 1997; Hill and Ferrara, 2000; Ferrara et al., 2003). Disruption of the GI mucosal barrier facilitates the translocation of immunostimulatory microbial products such as LPS into the systemic circulation (Hill et al., 1997; Cooke et al., 1998, 2001; Hill and Ferrara, 2000). LPS then stimulates mononuclear cells primed by donor T cell IFN- $\gamma$  to produce large amounts of inflammatory cytokines such as TNF and IL-1 and augments donor T cell activation, thereby potentiating both inflammatory and cellular effectors of GVHD (Nestel et al., 1992; Cooke et al., 1998, 2001). The administration of R-Spo1 protected against GI tract damage, leading to the fortification of GI tract mucosal barrier functions and reduction of the subsequent inflammatory milieu. An inflammatory environment would have further enhanced donor T cell activation (Nestel et al., 1992; Hill et al., 1997; Cooke et al., 1998), and R-Spo1 treatment



**Figure 5. R-Spo1 failed to attenuate GVHD in unirradiated host.** Unirradiated B6D2F1 mice were transplanted with  $12 \times 10^7$  splenocytes from syngeneic or allogeneic B6 donors. R-Spo1 (200  $\mu$ g/day) or control was administered from day -3 to -1 and day 1-3 after BMT. (A and B) Survival (A) and clinical GVHD scores (B; mean  $\pm$  SE) are shown. Syngeneic controls (Syn;  $n = 3$ ), allogeneic controls (control;  $n = 12$ ), and R-Spo1 ( $n = 12$ ) are shown. Data from two independent experiments were combined. (C-E) Numbers of donor CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen (C), CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes (D), and a complete blood count (E) on day 14 (Syn,  $n = 3$ ; control,  $n = 4$ ; R-Spo1,  $n = 4$ ) are shown. Data are representative of two independent experiments and are shown as mean  $\pm$  SD.



was also found to significantly reduce donor T cell proliferation and activation. As a result, brief administration of R-Spo1 modulates not only intestinal GVHD but also systemic GVHD. This study thus demonstrates for the first time that ISC damage plays a critical role in the exaggeration of GVHD.

The protective effects of R-Spo1 were not observed after allogeneic BMT in the absence of a conditioning regimen, thus suggesting a mechanism dependent on repair of conditioning-induced GI tract injury. In addition, R-Spo1 may act through different mechanisms before and after TBI; it protects best against systemic GVHD when administered before and after transplantation. Treatment with R-Spo1 before TBI expanded ISCs, suggesting an increased number of surviving ISCs that play a pivotal role in the regeneration of intestinal epithelium after injury. Additional administration of R-Spo1 posttransplant may further enhance proliferation and differentiation of the surviving ISCs, thereby allowing the regeneration of intestinal epithelium and fortification of mucosal barrier functions to suppress subsequent inflammatory milieu. It has been shown that a single ISC is sufficient for the reconstitution of a crypt-villus unit (Sato et al., 2009).

Reduction in the activation of donor T cells after BMT did not appear to be caused by the direct effect of R-Spo1 on T cells. A recent study has shown that  $\beta$ -catenin-transduced CD4<sup>+</sup>CD25<sup>+</sup> T reg cells survive longer than control cells, whereas  $\beta$ -catenin-transduced CD4<sup>+</sup> T cells become anergic (Ding et al., 2008). Wnt signaling arrests effector T cell differentiation by generating CD8<sup>+</sup> memory stem cells (Gattinoni et al., 2009). However, such changes were not apparent after BMT in our study. Wnt signaling is also important for hematopoiesis (Reya and Clevers, 2005); however, brief administration of R-Spo1 did not affect hematopoietic reconstitution after TCD BMT (unpublished data). We thus believe that R-Spo1 may preferentially stimulate ISCs rather than hematopoietic and T cells, as R-Spo1 transgenic mice show intestinal epithelial hyperplasia without any effects on lymphohematopoietic development (Kim et al., 2005). Alternately, such brief administration of R-Spo1 may not be sufficient to affect both the immune system and hematopoiesis. Wnt signaling has also been implicated in the pathogenesis of various tumors such as colon cancer and leukemia (Reya and Clevers, 2005; Román-Gómez et al., 2007). However, the incidence of tumorigenesis did not increase in R-Spo1 transgenic mice (Kim et al., 2006), and long-term treatment with R-Spo1 did not promote tumor xenograft growth in immunodeficient mice inoculated with various human colorectal tumor cell lines (Zhao et al., 2009). It thus follows that although caution should be exercised, it is unlikely that brief administration of R-Spo1 enhances tumorigenesis or the growth of preexisting tumors.

In summary, we found that ISCs are targets for GVHD and that protection of ISCs by R-Spo1 significantly improved the outcome of BMT by reducing systemic GVHD severity. By documenting that ISC damage is the key to this process, these results extend previous observations that the GI tract is not only a target organ for GVHD but also a crucial amplifier of systemic GVHD severity (Hill et al., 1998;

Panoskaltis-Mortari et al., 1998; Krijanovski et al., 1999; Teshima et al., 1999; Hill and Ferrara, 2000). An intensified conditioning regimen plays a critical role in controlling leukemia, but conditioning-related toxicity, particularly of the GI tract, limits the application of this curative therapy. Reduced intensity regimens have also been developed to explore the use of this therapy in older leukemic patients; however, better control of leukemia requires intensified conditioning in high-risk patients (Kahl et al., 2007). Thus, strategies to protect the GI tract from conditioning-related toxicity may allow safer application of intensified conditioning for controlling leukemia. Such a strategy has been tested in previous studies using IL-11 or keratinocyte growth factor. However, it is unfortunate that patients receiving IL-11 displayed severe fluid retention and early mortality (Antin et al., 2002), while keratinocyte growth factor failed to reduce conditioning regimen-mediated diarrhea (Blazar et al., 2006), thus making it impossible to further test the proposed strategy. R-Spo1 use is highly promising because of its direct, specific, and potent effects on ISCs; therefore, brief treatment with R-Spo1 may be used as an effective adjunct to clinical regimens of GVHD prophylaxis. This study presents a novel combined strategy for the rescue of both hematopoietic stem cells and ISCs in clinical medicine. Such a strategy may also be useful for treatment of other solid tumors and accidentally or intentionally irradiated victims, in whom damage to BM and the GI tract is a serious problem.

#### MATERIALS AND METHODS

**Mice and reagents.** Female B6 (H-2<sup>b</sup>, CD45.2<sup>+</sup>) and B6D2F1 (H-2<sup>b/d</sup>, CD45.2<sup>+</sup>) mice were purchased from Charles River, and B6-Ly5.1 (H-2<sup>b</sup>, CD45.1<sup>+</sup>) mice were obtained from the Jackson Laboratory. Mice were maintained as previously described (Teshima et al., 2002a). All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee. Recombinant human R-Spo1 was produced in CHO cells and purified as previously described (Zhao et al., 2007).

**BMT.** Mice were transplanted as previously described (Teshima et al., 2002a). In brief, after lethal TBI (x ray) delivered in two doses at 4-h intervals, B6D2F1 mice were intravenously injected with  $5 \times 10^6$  TCD BM cells with or without  $2 \times 10^6$  splenic T cells on day 0. Isolation of T cells and T cell depletion were performed using the T cell isolation kit and anti-CD90 microbeads, respectively, and AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. In some experiments, unirradiated B6D2F1 mice were intravenously injected with  $12 \times 10^7$  splenocytes.

**Assessment of GVHD.** Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by a scoring system that sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as described previously (Teshima et al., 2002a). Acute GVHD was also assessed by detailed histopathological analysis using a semiquantitative scoring system (Teshima et al., 2002a). Pictures from tissue sections were taken at room temperature using a digital camera (ProgRes 3012 mF; Jenoptik) mounted on a microscope (BX51; Olympus) and analyzed using a ProgRes PlugIn for PCI software version 5.0 (Jenoptik).

**Flow cytometric analysis.** mAbs used were FITC-, PE-, Cy5 PE-, or allophycocyanin-conjugated or biotinylated anti-mouse TCR- $\beta$ , IFN- $\gamma$ , CD4, CD8, CD25, CD45.1, CD45.2, CD44, CD62L, CD69, and B220 (BD),

and we also used Foxp3 (eBioscience). Surface marker staining and intracellular cytokine staining were performed as previously described (Teshima et al., 2002a; Asakura et al., 2010). At least 5,000 live samples were analyzed using FACSCalibur (BD) and FlowJo software (Tree Star, Inc.). The CFSE labeling of T cells was also performed as previously described (Teshima et al., 2002a).

**Immunohistochemical staining and in situ hybridization.** Slides were incubated at room temperature for 90 min with anti-mouse Ki-67 mAbs (Dako). We used Histofine Simple Stain MAXPO (rat) kits and subsequently diaminobenzidine solution (Nichirei) to generate brown-colored signals. Slides were then counterstained with hematoxylin. We measured villus height in 20 representative villi of the terminal ileum per slide as described previously (Farrell et al., 1998). For in situ hybridization, 1640-bp DNA fragments corresponding to nucleotide positions 17–435 of mouse *Olfm4* cDNA (GenBank/EMBL/DDJB accession no. NM\_001030294) were subcloned into pGEMT-Easy vectors (Promega) and used for generation of sense or antisense RNA probes. Digoxigenin-labeled RNA probes were prepared with DIG RNA labeling mix (Roche). Intestines were flushed, fixed in tissue fixative (Genostaff), embedded in paraffin, and sectioned at 6  $\mu$ m. Sections were then dewaxed, rehydrated, and digested with proteinase K solution, refixed, treated in acetic anhydride solution, and hybridized for 16 h at 60°C with probes at concentrations of 100 ng/ml in probe diluent (Genostaff). After washing, the sections were treated with 0.5% blocking reagent (Roche) in TBST (TBS with Tween 20) for 30 min and then incubated with antidigoxigenin alkaline phosphatase conjugate (Roche) diluted in a 1:1,000 ratio with TBST for 2 h. After washing, coloring reactions were performed with BM purple alkaline phosphatase substrate (Roche) overnight, and sections were then rewashed with PBS. Sections were then counterstained with Kernechtrot stain solution (Mutoh), dehydrated, and mounted with malinol (Mutoh).

**Cell cultures.** All culture media and incubation conditions have been previously described (Teshima et al., 2002b). Isolation of CD8<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells was performed by AutoMACS according to the manufacturer's instructions. Methods to generate DCs were previously described (Teshima et al., 2002b). T cells were cultured at a concentration of  $1 \times 10^5$  T cells/well with  $2.5 \times 10^3$  irradiated DCs/well or with 5  $\mu$ g/ml plate-bound anti-CD3 mAbs and 2  $\mu$ g/ml anti-CD28 mAbs. Supernatants were collected for measurement of cytokine levels 96 h after the initiation of culture, and cell proliferation was determined by thymidine uptake assay.

**ELISA.** For measuring IFN- $\gamma$  (BD) and TNF (R&D systems) levels, we performed ELISA according to the manufacturers' instructions with sensitivities of 31.25 pg/ml and 23.4 pg/ml, respectively. The Limulus amoebocyte lysate assay (Lonza) was performed according to the manufacturer's instructions to determine the serum level of LPS with a sensitivity of 0.1 EU/ml. All units expressed are relative to the US reference standard EC-2.

**Quantitative real-time PCR analysis.** Total RNA was purified using the RNeasy kit (QIAGEN). cDNA was synthesized using a QuantiTect reverse transcription kit (QIAGEN). PCR reactions and analyses were performed with ABI PRISM 7900HT SDS 2.1 (Applied Biosystems) using TaqMan Universal PCR master mix (Applied Biosystems), primers, and labeled TaqMan probes (TaqMan Gene Expression Assays; Applied Biosystems). The relative amount of each messenger RNA was determined using the standard curve method and was normalized to the level of GAPDH in each sample.

**Statistical analysis.** Mann-Whitney *U* tests were used to compare data, the Kaplan-Meier product limit method was used to obtain survival probability, and the log-rank test was applied to compare survival curves.  $P < 0.05$  was considered statistically significant.

**Online supplemental material.** Fig. S1 demonstrates that R-Spo1 stimulated proliferation of ISCs through the Wnt signaling pathway. Fig. S2

shows that R-Spo1 has no effects on proliferation and effector differentiation of T cells in response to CD3 or alloantigen stimulation in vitro. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20101559/DC1>.

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## Original article

**Analysis of immune reconstitution after autologous CD34<sup>+</sup> stem/progenitor cell transplantation for systemic sclerosis: predominant reconstitution of Th1 CD4<sup>+</sup> T cells****Hiroshi Tsukamoto<sup>1</sup>, Koji Nagafuji<sup>1</sup>, Takahiko Horiuchi<sup>1</sup>, Hiroki Mitoma<sup>1</sup>, Hiroaki Niiro<sup>1</sup>, Yojiro Arinobu<sup>2</sup>, Yasushi Inoue<sup>1</sup>, Kentaro To<sup>1</sup>, Toshihiro Miyamoto<sup>1</sup>, Hiromi Iwasaki<sup>2</sup>, Takanori Teshima<sup>2</sup>, Mine Harada<sup>1,3</sup> and Koichi Akashi<sup>1</sup>****Abstract****Objective.** The aim of this study is to evaluate the mechanism of long-term effect of autologous haematopoietic stem cell transplantation (ASCT) in treatment of SSc.**Methods.** Eleven patients (three males and eight females) with SSc were enrolled. Blood mononuclear cells were harvested after mobilization treatment with CYC and G-CSF. CD34<sup>+</sup> haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with  $>2 \times 10^6$ /kg autologous CD34<sup>+</sup> cells after high-dose CYC (50 mg/kg for 4 days) conditioning. Immune reconstitution was evaluated serially by analysing lymphocyte subpopulations for 36 months.**Results.** Progressive improvement of skin sclerosis has been observed for 3 years in most of the patients. The serum level of anti-Scl-70, an auto-antibody specific to SSc, was progressively decreased after ASCT. Improvement of skin sclerosis was significantly associated with the change in the serum anti-Scl-70 level after ASCT at 36 months. Serum levels of KL-6 and surfactant protein D, indicators for interstitial pneumonia activity, were also significantly decreased. The number of CD8<sup>+</sup> T cells immediately recovered within a month after ASCT, while the number of CD4<sup>+</sup> T cells remained low for  $>36$  months post-transplant. The majority of CD4<sup>+</sup> cells were memory but not naïve T cells, and regulatory CD4<sup>+</sup> T cells were not recovered. Th1/Th2 ratio was significantly increased after ASCT.**Conclusions.** ASCT with purified CD34<sup>+</sup> cells was effective in controlling the disease activity of SSc. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.**Key words:** Autoimmune disease, High-dose cyclophosphamide, Transplantation, Immune reconstitution, Th1/Th2 balance.**Introduction**

SSc is a heterogeneous autoimmune disease (AD) characterized by predominant T-cell activation, production

of auto-antibodies and cytokine release [1, 2]. All of these contribute to diffuse microvascular injury, leading to diffuse sclerosis within the skin and internal organs. DcSSc and internal organ involvement often cause life-threatening status of patients [3].

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Autologous haematopoietic stem cell transplantation (ASCT) was introduced for SSc treatment in 1996, and  $>100$  patients with SSc have been treated [4]. Phase I-II studies demonstrated that improvement of skin sclerosis and stabilization of interstitial pneumonia (IP) were achieved [5–10]. We also reported the safety and efficacy of high-dose CYC with ASCT for SSc patients [11]. The complete or partial remission of SSc was maintained for at least 3 years in these reports [6–9].

The working hypotheses of the efficacy of ASCT for SSc might be due to the immune reset that should include: (i) the eradication of auto-reactive lymphocytes by immunoablative conditioning; and/or (ii) the correction of dysregulated immune balance by newly developed lymphocytes derived from transplanted haematopoietic stem cells [12]. However, there remains controversy regarding whether such immune reset occurs in patients treated with ASCT. Muraro *et al.* [13] have reported that naïve CD4<sup>+</sup> T cells with diverse T-cell receptor repertoire, which were generated through thymus, developed after ASCT in patients with multiple sclerosis (MS). In contrast, in SSc patients, Farge *et al.* [14] reported the sustained reduction of CD4<sup>+</sup> T cells and B cells after ASCT.

Furthermore, Th1/Th2 balance might also be important in understanding the disease status of SSc. In a tight skin mouse, an animal model for SSc, the stimulation of Th1 immune responses prevents the development of scleroderma-like syndrome [15]. In human SSc, a shift from Th2 to Th1 responses is correlated with improvement of the skin fibrosis by longitudinal analysis of serum cytokine concentrations [16].

These data led us to analyse immune reconstitution in SSc patients treated with ASCT. In this study, we tracked reconstitution of T- and B-cell subpopulations after ASCT with purified CD34<sup>+</sup> haematopoietic stem/progenitor cells in SSc patients, and found that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. All patients showed sustained reduction of CD4 T cells, but Th1/Th2 ratio was increased during the 3-year observation. Thus, the clinical efficacy of ASCT might be dependent upon the skewed reconstitution of Th1 cells for a long time after ASCT.

## Patients and methods

The study was approved by the ethics committee of Kyushu University Hospital. Written informed consent was obtained from all patients according to the Declaration of Helsinki.

### Patients and eligibility

Eligibility of patients with SSc for ASCT was previously described [11]. Briefly, patients with SSc were eligible when they had severe diffuse SSc that had rapidly developed over the previous 4 years. They also had to have at least one of the following organ involvements: (i) pulmonary, (ii) cardiac or (iii) renal involvement. Patients with limited SSc were considered eligible when progressive and life-threatening IP was present. Patients were excluded from the study when they had uncontrolled arrhythmia, severe heart failure, pulmonary hypertension, DL<sub>CO</sub> <20% of predicted and renal failure as previously described [11]. All patients were followed up for at least 36 months after transplantation for the evaluation of immune reconstitution. Blood was obtained from healthy donors (after informed consent, *n* = 10) to determine the reference values of lymphocyte subpopulations and Th1/Th2

balance, and from SSc patients (after informed consent, *n* = 13) for FoxP3 staining.

### Peripheral blood stem cell collection and ASCT

Peripheral blood stem cells (PBSCs) were mobilized during haematologic recovery after relatively high-dose CYC (2 g/m<sup>2</sup>) for 2 days followed by administration of recombinant human G-CSF (filgrastim; Kirin Brewery, Tokyo, Japan) as previously described [11]. After collecting PBSCs to obtain 2 × 10<sup>6</sup> CD34<sup>+</sup> cells/kg or more by apheresis, CD34<sup>+</sup> cells were positively selected using an immunomagnetic bead with an anti-CD34 mAb (CliniMACS; Miltenyi Biotec, Germany). For pre-transplant conditioning, high-dose CYC (50 mg/kg) was given for 4 days from Day -5 to -2 and frozen-thawed CD34<sup>+</sup> cells were transplanted on Day 0. Patients 1, 2, 3, 4 and 6 received G-CSF from Day 1 [11]. Concomitantly administered prednisolone doses were kept unchanged or gradually tapered according to the individual clinical status after ASCT. The concomitantly administered immunosuppressants were stopped before the administration of CYC for mobilization.

### Treatment outcome

The modified Rodnan skin score (mRSS) was used to evaluate the improvement of skin sclerosis when initial mRSS was ≥15 [11]. Serum levels of anti-Scl 70 (anti-topo I) antibody were measured by an ELISA kit (MBL, Nagoya, Japan). Serum levels of KL-6 and surfactant protein D (SP-D) were measured by ELISA kits (Sanko Junyaku Co., Tokyo and Yamasa Co., Choshi, respectively, Japan). Serum levels of immunoglobulin were measured by laser nephelometry.

### Lymphocyte phenotyping

Lymphocyte immunophenotyping of samples from whole blood was performed by IF flow cytometry before mobilization, before haematopoietic stem cell transplantation (HSCT) and 1, 3, 6, 12, 18, 24, 36 months after HSCT. The following mAbs and their combinations were used: anti-CD3-FITC; anti-CD19-phycoerythrin (PE); anti-CD20-FITC; anti-CD4-FITC; anti-CD8-PE; anti-CD45RA-PE; anti-CD45RO-PE; anti-CD25-PE; anti-CD16-FITC; anti-CD56-PE, anti-CD69-FITC and anti-CD27-FITC (eBioscience, San Diego, CA, USA). The CD4<sup>+</sup>FoxP3<sup>+</sup> cells were detected by using anti-CD4-FITC, anti-FoxP3-PE and permeabilization buffer (eBioscience) in 9 patients at 37.8 (18.0) months (one sample for each patient) after ASCT and 13 SSc controls without ASCT. Results were expressed as the absolute number of cells.

### Th1/Th2 balance

Flow cytometric determination of IFN-γ and IL-4 in the cytoplasm of peripheral CD4<sup>+</sup> T cells was performed by a previously described method [17]. Briefly, aliquots (500 μl) of heparinized whole blood were stimulated with a combination of 25 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin in the presence of brefeldin A (Sigma, St Louis, MO, USA) and cultured for 4 h at 37°C

in a humidified incubator containing 7% CO<sub>2</sub>. Activated cultures were aliquoted and stained with 20 µl of peridinin chlorophyll protein-conjugated CD4-specific mAb (Becton Dickinson, San Jose, CA, USA) for 15 min at room temperature, and then treated with 2 ml of FACS lysing solution (Becton Dickinson). After a short incubation (5 min), the samples were centrifuged and combined with FACS permeabilizing solution (Becton Dickinson) for 10 min at room temperature in the dark. The sample tubes were washed twice and FITC-conjugated IFN-γ-specific mAb and PE-conjugated IL-4-specific mAb (Becton Dickinson) for 30 min at room temperature in the dark. FITC-conjugated mouse IgG2a and PE-conjugated mouse IgG1 were used as controls. After washing again, the cells were resuspended in 1% paraformaldehyde and analysed by flow cytometry. The percentage of IFN-γ or IL-4-positive cells (percentage of IFN-γ or percentage of IL-4) was counted by FACS, and Th1/Th2 balance was evaluated by a ratio of percentage of IFN-γ to percentage of IL-4 (IFN-γ/IL-4).

#### Cytokine and chemokine levels

Serum levels of TNF-α and TGF-β were measured with sandwich ELISA kits (Quantikine; R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions before and after ASCT. Serum levels of IL-6 and soluble receptor for IL-2 (sIL-2R) were measured with CLISA kit (Fujirebio, Tokyo, Japan) and with ELISA kit (Kyowamedix, Tokyo, Japan), respectively.

#### Statistical analysis

Values were expressed as mean (s.d.). Student's *t*-test was used for statistical analysis of the data. The correlations of mRSS with the level of anti-Scl-70 or Th1/Th2 balance were investigated by Pearson's correlation coefficient test. Difference with *P* < 0.05 was considered to be statistically significant.

TABLE 1 Patient profile and number of reinfused cells

Patient number	Sex	Age, years	mRSS	IP	%VC/DL <sub>CO</sub>	Anti-Scl-70	Prior therapy	Follow-up, month	Number of reinfused CD34 <sup>+</sup> cells (×10 <sup>9</sup> /kg)	Number of reinfused CD3 <sup>+</sup> cells (×10 <sup>4</sup> /kg)
1	F	54	16	+	58/51	+	St, CYC	88	8.4	0.33
2	M	55	15	+	65/47	+	St	78	4.9	0.27
3	M	58	31	+	63/44	–	St	77	2.2	2.95
4	F	54	26	+	73/60	+	St	75	2.1	1.71
5	F	53	28	+	74/29	+	St	72	7.2	3.0
6	F	48	32	+	77/25	+	St, CYC, CsA	68	4.0	2.35
7	F	34	8	+	39/ND	–	–	20	9.1	1.03
8	F	61	26	+	90/87	+	St	52	3.0	0.32
9	F	61	32	+	56/37	+	St, CsA	47	4.2	0.13
10	F	44	15	+	45/35	+	St, CY	43	4.9	0.0
11	M	53	7	+	74/44	+	St	39	1.3	0.26
Ave. (s.d.)		52.3 (7.5)	21.5 (9.0)		64.9 (14.8)/45.8 (18.0)			60.0 (21.0)	4.7 (2.6)	1.1 (1.2)

F: female; M: male; Ave.: Average; ND: not done.

## Results

Autologous transplantation with purified CD34<sup>+</sup> mobilized blood stem cells was successfully performed without treatment-related mortality

Eleven patients, eight females and three males, were enrolled in this study. Clinical results of Patients 1–6 at 12 months after ASCT were previously reported [11]. The mean (s.d.) of mRSS was 21.5 (9.0), and all patients developed clinical IP with decreased per cent vital capacity (VC) and per cent DL<sub>CO</sub> [mean (s.d.), 64.9 (14.8) and 45.8 (18.0)%, respectively] (Table 1). The anti-Scl-70 antibody, an auto-antibody specific to diffuse SSc, was detected in 9 out of 11 patients. All patients were resistant to the standard treatment with CS, CYC and/or ciclosporin. Blood mononuclear cells were harvested after mobilization with CYC and G-CSF. CD34<sup>+</sup> haematopoietic stem/progenitor cell fractions were purified and cryopreserved. Patients were transplanted with purified autologous CD34<sup>+</sup> cells after high-dose CYC (500 mg/kg) conditioning. Consequentially, 4.7 × 10<sup>9</sup>/kg CD34<sup>+</sup> cells with 1.1 (1.2) × 10<sup>4</sup>/kg CD3<sup>+</sup> T-cell contaminants were transplanted. ASCT was successfully performed without transplantation-related mortality in all patients, but Patient 7 died due to the progression of IP at 20 months after ASCT. The clinical status of the remaining 10 patients was followed up for >36 months. There was a variety of post-transplant infections such as adenoviral haemorrhagic cystitis, herpes zoster and cytomegaloviral antigenaemia [18].

### CD34<sup>+</sup> ASCT treatment induced resolution of refractory SSc

The effect of ASCT on skin sclerosis was assessed by the change in mRSS. After ASCT, skin sclerosis was progressively resolved, and had improved by 72.0% at 36 months post-transplant (Fig. 1A). The vital capacity of the lung was increased from 64.9 to 77.8% at 36 months, although

DL<sub>CO</sub> was unchanged (Fig. 1B). Serum levels of KL-6 and SP-D, indicators of IP activity [19, 20], were significantly decreased (Fig. 1C and D).

Reflecting the resolution of clinical symptoms of SSc, the serum level of anti-Scl-70 progressively decreased after ASCT (Fig. 2A), independent of serum immunoglobulin levels (Fig. 2B). A significant correlation ( $r=0.52$ ,  $P<0.05$ ) was observed between the change in mRSS and that in the serum level of anti-Scl-70 at 36 months after ASCT.

Serum levels of pro-inflammatory or pro-fibrotic cytokines and a T-cell activation marker such as TNF- $\alpha$ , TGF- $\beta$ , IL-6 and soluble IL-2R (sIL-2R), were also decreased after ASCT (Fig. 3A-D). These data show that ASCT with purified CD34<sup>+</sup> cells is effective in controlling disease activity of SSc patients refractory to conventional immunosuppressive therapies.

The recovery of CD4<sup>+</sup> T cells was particularly retarded after ASCT with purified CD34<sup>+</sup> cells in SSc patients

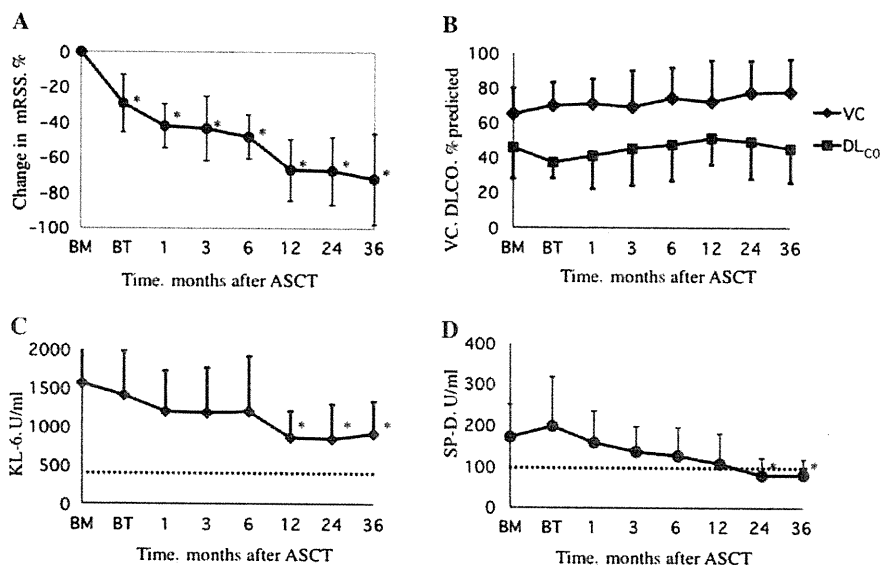
Because the patients were infused with highly purified CD34<sup>+</sup> haematopoietic stem/progenitor cells containing very low numbers of lymphocytes, we tracked the immune reconstitution after ASCT by analysing lymphocyte subpopulations for 36 months.

As shown in Fig. 4A, although the absolute number of CD8<sup>+</sup> T cells returned to the normal level only a month after ASCT, the recovery of CD4<sup>+</sup> T cells was apparently retarded; at 36 months post-transplant, the number of

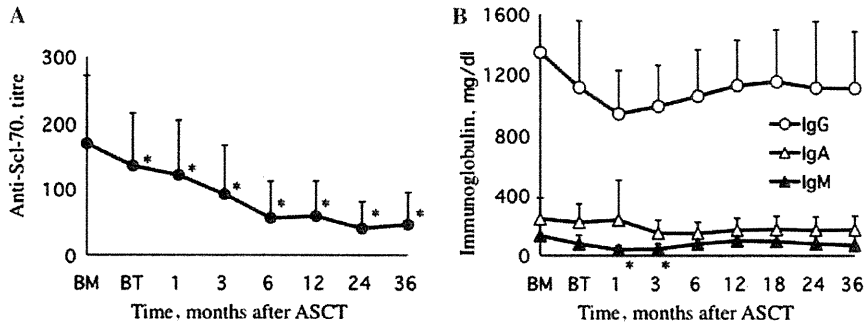
CD4<sup>+</sup> cells remained below the normal range, resulting in continuous inversion of the CD4/CD8 ratio. Previous reports have shown that the long-term remission after ASCT in MS and SLE was associated with the increased number of thymus-derived naïve CD4<sup>+</sup> T cells [13, 21]. Therefore, we analysed functional subsets of CD4<sup>+</sup> T cells. As shown in Fig. 4B, the number of memory CD4<sup>+</sup>CD45RO<sup>+</sup> T cells was significantly decreased for 1–3 months after CD34<sup>+</sup> ASCT, but returned to the baseline level 24 months after ASCT, although it did not reach the normal range. In contrast, naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were significantly decreased after mobilization, and remained at a low level at 36 months post-ASCT. These data show that most recovered CD4<sup>+</sup> T cells were memory cells but not naïve T cells. The CD4<sup>+</sup>CD25<sup>+</sup> T cell fraction that includes activated and regulatory T cells [22] disappeared after ASCT, and remained low in number even at 36 months post-ASCT (Fig. 4C). The number of CD4<sup>+</sup>FoxP3<sup>+</sup> cells in 9 patients at 37.8 (18.0) months after ASCT was significantly lower than that of 13 SSc controls [8.06 (7.61) vs 27.23 (13.30)/ $\mu$ l,  $P<0.05$ ]. These results show that although the patients displayed resolution of clinical SSc after ASCT, naïve CD45RA<sup>+</sup> T cells or regulatory T cells did not reconstitute well after ASCT.

SSc patients have distinct abnormalities of B-cell subpopulations, characterized by expanded naïve B cells and activated but diminished memory B cells [23]. The number of CD19<sup>+</sup> B cells returned into the normal range 18 months after ASCT.

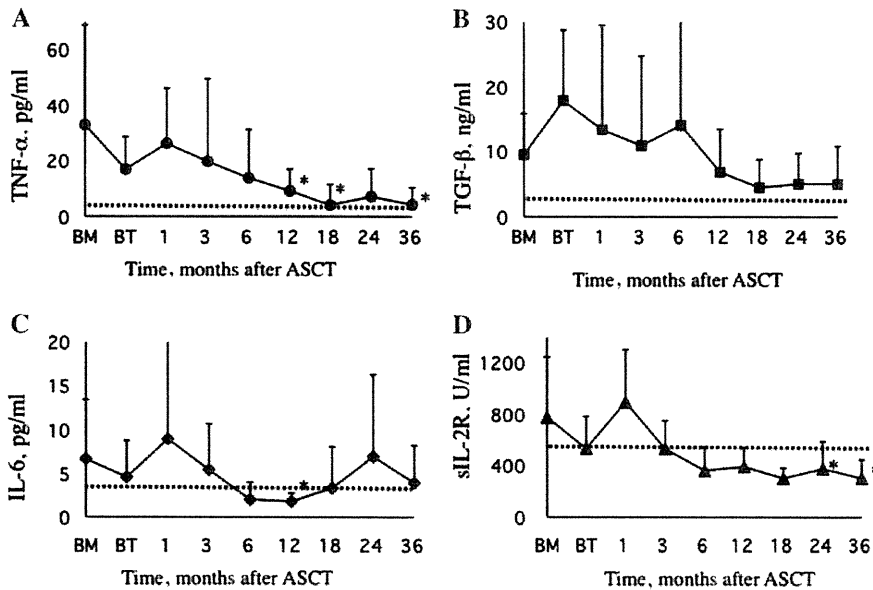
**Fig. 1** Resolution of SSc after ASCT. **(A)** mRSS. The proportional change from baseline measurement was calculated for each time point. Patients with an initial mRSS of  $\geq 15$  were included. **(B)** VC and DL<sub>CO</sub>. **(C)** KL-6. **(D)** SP-D. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. \* $P<0.05$  vs BM. (C and D) Dashed line shows upper normal limit.



**Fig. 2** Evaluation of autoantibody and immunoglobulin. **(A)** Change in the titre of anti-Scl-70. **(B)** Change in the serum levels of IgG, IgA and IgM. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. \**P* < 0.05 vs BM.



**Fig. 3** Kinetics of immunological markers in SSc patients who received ASCT. Data are mean (s.d.). The x-axis is not drawn to scale. **(A)** TNF- $\alpha$ , **(B)** TGF- $\beta$ , **(C)** IL-6, **(D)** sIL-2R. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. \**P* < 0.05 vs BM. Dashed line shows upper normal limit.



To study whether correction of abnormalities in functional subsets of B cells was associated with durable remission, we focused on the functional subset of CD19<sup>+</sup> B cells. The number of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells was low at baseline, which was characteristic of SSc [23]. An increase in the number of memory B cells, however, was not observed even at 36 months after ASCT, and the vast majority of recovered B cells were naïve CD19<sup>+</sup>CD27<sup>-</sup> B cells (Fig. 4D).

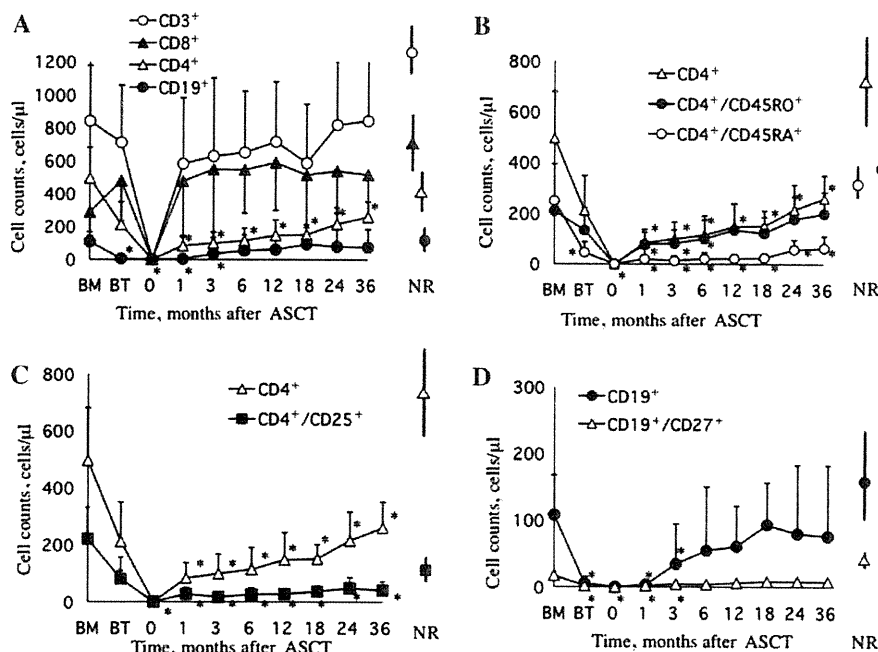
Th1 CD4<sup>+</sup> T cells were predominantly reconstituted after CD34<sup>+</sup> ASCT

Previous data showed that Th1/Th2 balance was associated with skin sclerosis in an animal model of SSc

[15]. Therefore, we analysed Th1/Th2 balance after ASCT by measuring the ratio of intracellular IFN- $\gamma$ <sup>+</sup>/IL-4<sup>-</sup>/CD4<sup>+</sup> to IFN- $\gamma$ <sup>-</sup>/IL-4<sup>+</sup>/CD4<sup>+</sup> T cells (Fig. 5). In Fig. 5A, the results of Case 9 before mobilization, 3 and 24 months after HSCT were shown as representative data. IFN- $\gamma$ <sup>+</sup>/IL-4<sup>-</sup>/CD4<sup>+</sup> and IFN- $\gamma$ <sup>-</sup>/IL-4<sup>+</sup>/CD4<sup>+</sup> T cells were considered to be Th1 and Th2 cells, respectively. In this case, elimination of IL-4<sup>+</sup>/CD4<sup>+</sup> (Th2) T cells as well as predominant reconstitution of IFN- $\gamma$ <sup>+</sup>/CD4<sup>+</sup> (Th1) T cells were observed after ASCT. The ratio of IFN- $\gamma$ <sup>+</sup> to IL-4<sup>+</sup> CD4<sup>+</sup> T cells was increased from 4.3 before mobilization to 32.8 at 3 months and 197.3 at 24 months after HSCT. When Th1/Th2 balance was analysed in all SSc patients, the ratio of IFN- $\gamma$ <sup>+</sup> to IL-4<sup>+</sup>/CD4<sup>+</sup> T cells was significantly



**Fig. 4** Immune reconstitution after ASCT. Change in absolute cell counts of immune cells. **(A)** CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup> cells. **(B)** CD4<sup>+</sup>, CD4<sup>+</sup>CD45RO<sup>+</sup>, CD4<sup>+</sup>CD45RA<sup>+</sup> cells. **(C)** CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup> cells. **(D)** CD19<sup>+</sup>, CD19<sup>+</sup>CD27<sup>+</sup> cells. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. \**P* < 0.05 vs BM. Normal ranges (NR: 95% CI) are shown as right-sided vertical bars.



increased at 1 month and reached a plateau at 6 months after ASCT (Fig. 5B). The skewed reconstitution of Th1 CD4<sup>+</sup> T cells was maintained for 36 months after HSCT. There were no significant correlations between the changes in mRSS and those in Th1/Th2 balance.

## Discussion

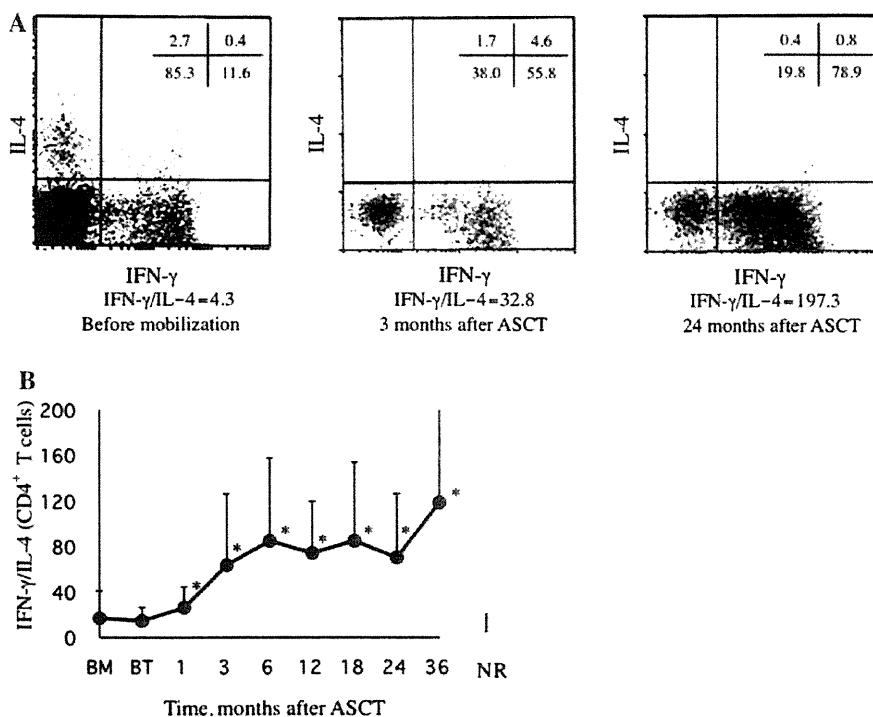
In this study, the resolution of disease was progressively obtained in SSc patients for 36 months after ASCT. This durable effect was not due to the reconstitution of naïve CD4<sup>+</sup> T cells, regulatory T cells or the correction of B-cell imbalance. On the other hand, the elimination of Th2 cells by high-dose CYC as well as the predominant reconstitution of Th1 cells were observed after ASCT. Reflecting the resolution of clinical symptoms of SSc, serum levels of anti-Scl-70 progressively decreased after ASCT. Serum levels of KL-6 and SP-D, indicators for IP activity, were also significantly decreased.

In patients with SSc, production levels of type 2 cytokines such as IL-4, IL-6 and IL-13 by stimulated peripheral blood mononuclear cells and cultured CD4<sup>+</sup> T cells decreased [24, 25]. Our data showed that the ratio of IFN- $\gamma$ - to IL-4-producing CD4<sup>+</sup> T cells was significantly increased in a month and was sustained for 36 months after ASCT. The predominant reconstitution of IFN- $\gamma$ -producing cells is associated with amelioration of skin

sclerosis, probably due to an ability of IFN- $\gamma$  to reduce excessive collagen synthesis by scleroderma-derived fibroblasts [26]. IL-4 increases collagen production of fibroblasts and induces the production of TGF- $\beta$  in patients with SSc [27]. Therefore, the elimination of IL-4-producing T cells provides a favourable effect on SSc. The limitation of this study was small sample size and that there were not significant correlations between the changes in mRSS and those in Th1/Th2 balance. It is unclear how predominant reconstitution of Th1 CD4<sup>+</sup> T cells after ASCT is induced. Polarization of CD4<sup>+</sup> T cells after ASCT may depend on the local levels of cytokines such as IL-12 or IL-4 when naïve CD4<sup>+</sup> T cells develop into functional T cells [28]. Predominant reconstitution of Th1 CD4<sup>+</sup> T cells after ASCT may also occur in patients with other autoimmune diseases when treated by ASCT. Therefore, it is conceivable that ASCT is potentially effective for Th2-related diseases such as SSc and SLE, while its effect on Th1-related diseases such as RA is limited [4]. Macrophage activation syndrome is often observed in patients with JIA after ASCT [29]. It may be associated with a Th1 immune response after ASCT.

Our data showed that despite the resolution of clinical symptoms of SSc, patients did not achieve normalization of lymphocyte compartment, even 3 years after ASCT. The recovery of CD4<sup>+</sup> T cell was delayed until 36 months after ASCT. Muraro *et al.* [13] reported that

**Fig. 5** Evaluation of Th1/Th2 balance after ASCT. **(A)** Representative expression of IFN- $\gamma$  and IL-4 in the cytoplasm of CD4<sup>+</sup> T cells (Case 9) before mobilization, 3 months and 24 months after ASCT. IFN- $\gamma$ /IL-4 was defined as the ratio of IFN- $\gamma^+$ /IL-4<sup>-</sup> to IFN- $\gamma^-$ /IL-4<sup>+</sup>. Number of analysed cells was decreased after ASCT since ratio of CD4<sup>+</sup> T cells in gated cells was decreased after ASCT. **(B)** Change in the ratio of intracellular IFN- $\gamma^+$  to IL-4<sup>+</sup> CD4<sup>+</sup> T cells after ASCT in patients with SSc. Data are presented as mean (s.d.). The x-axis is not drawn to scale. The data obtained before mobilization and just before transplantation (HSCT) are shown as BM and BT, respectively. \**P* < 0.05 vs BM. Normal range (NR: 95% CI) is shown as right-sided vertical bar.



naïve CD45RA<sup>+</sup> T cells with diverse TCR repertoire and of thymic origin, were increased after ASCT in patients with MS, and that the increase of such cells was associated with long-term suppression of inflammatory activity of MS. In contrast, the present study revealed that the recovery of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells was so severely suppressed for 36 months after HSCT and that most of the recovered CD4<sup>+</sup> T cells were memory CD45RO<sup>+</sup> T cells (Fig. 4B). This discrepancy of T-cell recovery after ASCT between SSc and MS, may be due to the difference of disease and/or of age at inclusion. In the study of Farge *et al.* [14], the level of naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells was also suppressed for 9 months after ASCT in SSc patients. In the study of Storek *et al.* [30], naïve and memory CD4<sup>+</sup> T cells were equally recovered in 24 months after ASCT in patients with MS or SSc.

CD25<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells are a major regulator of adaptive immunity [22]. Patients with JIA showed a significant increase in thymus-derived regulatory T cells (CD25<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>) following ASCT [22]. However, in this study, the recovery of CD25<sup>+</sup>CD4<sup>+</sup> T cells was severely delayed compared with that of CD25<sup>-</sup>CD4<sup>+</sup> T cells

(Fig. 4C); the number of CD25<sup>+</sup>CD4<sup>+</sup> T cells did not reach the lower limit of normal even at 36 months after ASCT. It is unlikely that the number of regulatory T cells was increased after ASCT, even if we considered that CD25<sup>+</sup>CD4<sup>+</sup> T cells included activated T cells as well as regulatory T cells. When we analysed CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in nine patients with SSc, their recovery after ASCT was retarded. These results show that the expansion of regulatory T cells after ASCT was not the cause of the efficacy of ASCT on SSc.

The number of CD19<sup>+</sup>CD27<sup>+</sup> memory B cells was low in contrast to an increased number of CD19<sup>+</sup>CD27<sup>-</sup> naïve B cells at the baseline. Sato *et al.* [23] reported the B-cell abnormality including the expanded naïve B cells and diminished memory B cells in SSc patients. Unexpectedly, recovery of memory CD19<sup>+</sup>CD27<sup>+</sup> B cells was severely suppressed even at 36 months after ASCT (Fig. 4D). In the study of Storek *et al.* [30], both naïve and memory B cells recovered to the normal range in 6 months after ASCT. Collectively, the resolution of clinical SSc after ASCT was not due to the reconstitution of naïve CD4<sup>+</sup> T cells or to that of regulatory T cells or to the correction of B-cell imbalance.

An anti-Scl-70 antibody, a useful marker in establishing the diagnosis of SSc, predicts diffuse skin involvement and pulmonary fibrosis, and the increased level of this antibody is associated with a poor prognosis. In this study, we, for the first time, showed that the level of an anti-Scl-70 antibody was continuously decreased for 36 months after ASCT, and that the changes in anti-Scl-70 level were correlated significantly with those in mRSS. These results are consistent with a previous report that showed the correlation of serum anti-Scl-70 levels with disease activity in SSc [31], although the role of anti-Scl-70 in the pathogenesis of SSc was not clearly demonstrated in this article. It is of interest that the changes in serum anti-Scl-70 levels were independent of those in serum immunoglobulin levels, which returned to the baseline level at 12 months after ASCT (Fig. 2). In the study of Storek *et al.* [30], the level of anti-Scl-70 continued to be abnormally high throughout 24 months after ASCT. This difference might come from the difference in transplant conditioning (CYC 200 vs CYC 120 mg/kg + total body irradiation 8 Gy + anti-thymocyte globulin) or in the purity of the CD34<sup>+</sup> cells.

Dysregulated cytokine production was reported in SSc patients [32, 33]. In this study, serum levels of TNF- $\alpha$ , TGF- $\beta$ , IL-6 and sIL-2R increased before mobilization as previously reported [32], but their levels significantly decreased after ASCT (Fig. 3). Serum levels of VEGF and monocyte chemotactic protein 1 (MCP-1), however, did not decrease after ASCT (data not shown). The decreased levels of profibrotic cytokines after ASCT might reflect resolution of the disease.

Patient 7 died due to progressive IP in spite of the improvement of skin sclerosis at 20 months after ASCT. In this patient, IP was already highly advanced (per cent VC 39%) at the time of ASCT. Immune reconstitution after ASCT was similarly obtained in terms of Th1/Th2 balance and serum levels of pro-fibrotic cytokines. Therefore, the disease was fatal because of advanced and refractory IP that did not respond to the resolution of autoimmune reactions. This result suggests that patients with advanced organ involvement need to be excluded in a future study.

In conclusion, ASCT with purified CD34<sup>+</sup> cells was effective in controlling the disease activity of SSc. Improvement of skin sclerosis was significantly associated with the change in serum anti-Scl-70 level after ASCT. Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

#### Rheumatology key messages

- ASCT causes durable remission in patients with SSc.
- Th1/Th2 ratio was significantly increased for at least 3 years after ASCT.

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